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☐ 2. 6268131. 15 Dec 97; 31 Jul 01. Mass spectrometric methods for sequencing nucleic acids. Kang; Changwon, et al. 435/6; 435/91.2. C12Q001/68.

☐ 3. 6194148. 06 Feb 98; 27 Feb 01. Method for detecting a hybridized nucleic acid molecule. Hori; Kunio, et al. 435/6; 435/91.2 536/23.1 536/24.3. C12Q001/68 C12P019/34 C07H021/02 C07H021/04.

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END.DWPI,EPAB,JPAB,USPT.	4400503
ENDA.DWPI,EPAB,JPAB,USPT.	325
ENDB.DWPI,EPAB,JPAB,USPT.	19
ENDC.DWPI,EPAB,JPAB,USPT.	35
ENDD.DWPI,EPAB,JPAB,USPT.	38
ENDE.DWPI,EPAB,JPAB,USPT.	616
ENDF.DWPI,EPAB,JPAB,USPT.	49
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(3 AND EXONUCLEASE$1).USPT,JPAB,EPAB,DWPI.	9
(L3 AND EXONUCLEASE$1).USPT,JPAB,EPAB,DWPI.	9

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L3: Entry 12 of 14 File: USPT Nov 28, 1995

DOCUMENT-IDENTIFIER: US 5470705 A
TITLE: Probe composition containing a binding domain and polymer chain and methods of use

Brief Summary Paragraph Right (6):
Another approach to detecting the presence of a given sequence or sequences in a polynucleotide sample involves selective amplification of the sequence(s) by polymerase chain reaction (Mullis, Saiki). In this method, primers complementary to opposite end portions of the selected sequence(s) are used to promote, in conjunction with thermal cycling, successive rounds of primer-initiated replication. The amplified sequence may be readily identified by a variety of techniques. This approach is particularly useful for detecting the presence of low-copy sequences in a polynucleotide-containing sample, e.g., for detecting pathogen sequences in a body-fluid sample.

Brief Summary Paragraph Right (7):
More recently, methods of identifying known target sequences by probe ligation methods have been reported (Wu, Whiteley, Lundegren, Winn-Deen). In one approach, known as oligonucleotide ligation assay (OLA), two probes or probe elements which span a target region of interest are hybridized with the target region. Where the probe elements match (basepair with) adjacent target bases at the confronting ends of the probe elements, the two elements can be joined by ligation, e.g., by treatment with ligase. The ligated probe element is then assayed, evidencing the presence of the target sequence.

Brief Summary Paragraph Right (15):
In one general method, each probe includes first and second probe elements having first and second sequence-specific oligonucleotides which, when bound in a sequence specific manner to a selected single-stranded target sequence, have (or can be modified to have) confronting end subunits which can basepair to adjacent bases in the target polynucleotide sequence. After hybridizing the oligonucleotides to the target polynucleotide, the target-bound oligonucleotides are ligated, to join those hybridized oligonucleotides whose confronting end subunits are base-paired with adjacent target bases. In each pair of probe elements, one of the probe elements contains the probe-specific polymer chain, and the other element preferably includes a detectable reporter.

Brief Summary Paragraph Right (16):
In a second general embodiment, each probe includes first and second primer elements having first and second sequence-specific oligonucleotide primers effective to hybridize with opposite end regions of complementary strands of a selected target polynucleotide segment. The first probe element contains the probe-specific polymer chain. The primer elements are reacted with the target polynucleotide in a series of primer-initiated polymerization cycles which are effective to amplify the target sequence of interest.

Brief Summary Paragraph Right (18):
In a third general embodiment, bound oligonucleotide probes are reacted with reporter-labeled nucleoside triphosphate molecules, in the presence of a DNA polymerase, to attach reporter groups to the 3' end of the probes.

Brief Summary Paragraph Right (19):
In a fourth general embodiment, each probes includes a binding polymer which is modified by enzymatic cleavage when bound to a target sequence. The cleavage reaction may remove a portion of the binding polymer, to modify the probes's ratio of charge/translational frictional drag, or may separate a reporter label carried at one

end of the binding polymer from a polymer chain carried at the other end of the binding polymer, to modify the charge/translational frictional drag of the binding polymer carrying the reporter label.

Brief Summary Paragraph Right (23):
In another embodiment, each sequence specific probe in the composition further includes a second binding polymer, where the first-mentioned and second binding polymers in a sequence-specific probe are effective to bind in a base-specific manner to opposite end regions of opposite strands of a selected duplex target sequence, allowing primer initiated polymerization of the target region in each strand. The second binding polymer preferably includes a detectable label, and the polymer chain attached to the first binding polymer imparts to each ligated probe pair, a distinctive combined ratio of charge/translational frictional drag.

Drawing Description Paragraph Right (5):
FIGS. 4A and 4B illustrate coupling reactions for coupling the polymer chains of FIGS. 2 and 3 to the 5' end of a polynucleotide, respectively. The nucleic acid sequence given in FIGS. 4A and 4B is presented as SEQ ID NO:1;

Detailed Description Paragraph Right (19):
The oligonucleotide binding polymer in probe 20 is derivatized, at its 5' end, with a polymer chain 27 composed of N subunits 28. The units may be the subunits of the polymer or may be groups of subunits. Exemplary polymer chains are formed of polyethylene oxide, polyglycolic acid, polylactic acid, polypeptide, oligosaccharide, polyurethane, polyamids, polysulfonamide, polysulfoxide, and block copolymers thereof, including polymers composed of units of multiple subunits linked by charged or uncharged linking groups.

Detailed Description Paragraph Right (24):
In one preferred embodiment, described below, the polymer chain is formed of hexaethylene oxide (HEO) units, where the HEO units are joined end-to-end to form an unbroken chain of ethylene oxide subunits, as illustrated in FIG. 2, or are joined by charged (FIG. 5) or uncharged (FIG. 3) linkages, as described below. Other embodiments exemplified below include a chain composed of N 12mer PEO units, and a chain composed of N tetrapeptide units.

Detailed Description Paragraph Right (27):
FIG. 1B illustrates a probe 25 which has a sequence-specific oligonucleotide binding polymer 21 designed for sequence-specific, i.e., base-specific binding to a region of a target polynucleotide 23. By this is meant the binding polymer contains a sequence of subunits effective to form a stable duplex or triplex hybrid with the selected single-stranded or double-stranded target sequence, respectively, under defined hybridization conditions. As will be seen with reference to FIG. 17 below, the binding polymer may contain both DNA and RNA segments. Attached to the binding polymer, at its 5' end, is a polymer chain 31 composed of N units 33, which imparts to the binding polymer a distinctive ratio of charge/translational frictional drag, as described above. The 3' end of the binding polymer is derivatized with a reporter or tag 39. In one aspect, the invention includes a composition which includes a plurality of such probes, each with a different-sequence binding polymer targeted against different target regions of interest, and each with a distinctive ratio of charge/translational frictional drag imparted by the associated polymer chain.

Detailed Description Paragraph Right (29):
In the embodiment illustrated, the sequences of interest may involve mutations, for example, point mutations, or addition or deletion type mutations involving one or a small number of bases. In a typical example, the expected site of mutation is near the midpoint of the known-sequence target region, and divides that region into two subregions. In the example shown, the mutation is a point mutation, and the expected site of the mutation is at one of the two adjacent bases T-G, with the T base defining the 5' end of a subregion 38a, and the adjacent G base, defining the 3' end of adjacent subregion 38b. As will be seen below, the probe elements are also useful for detecting a variety of other types of target sequences, e.g., sequences related to pathogens or specific genomic gene sequences.

Detailed Description Paragraph Right (30):

Probe element 32, which is representative of the first probe elements in the probe composition, is composed of an oligonucleotide binding polymer element 42 which preferably includes at least 10-20 bases, for requisite basepair specificity, and has a base sequence which is complementary to a subregion 38a in the target polynucleotide. In particular, the 3' end nucleotide bases are selected for base pairing to the 5' end nucleotide bases of the corresponding subregion, e.g., the A:T matching indicated.

Detailed Description Paragraph Right (31):
The oligonucleotide in the first probe element is derivatized, at its 5' end, with a polymer chain 44 composed of N preferably repeating units 45, substantially as described with respect to chain 27 formed from units 28. As described with respect to probe 20, the polymer chain in the first probe element imparts a ratio of charge/translational frictional drag which is distinctive for each sequence-specific probe element in the composition.

Detailed Description Paragraph Right (32):
Second probe element 36, which is also representative of the second probe elements in the probe composition, is composed of an oligonucleotide polymer binding element 46 which preferably includes at least 10-20 bases, for requisite basepair specificity, and has a base sequence which is complementary to a subregion 38b in the target polynucleotide. In particular, the 5' end nucleotide bases are selected for base pairing to the 3' end nucleotide bases of the corresponding subregion, e.g., the C:G matching indicated.

Detailed Description Paragraph Right (33):
As seen in FIG. 1C, when the two probe elements are both hybridized to their associated target regions, the confronting end subunits in the two probes, in this example the confronting A and C bases, are matched with adjacent bases, e.g., the adjacent T and G bases in the target polynucleotide. In this condition, the two probe elements may be ligated at their confronting ends, in accordance with one embodiment of the invention described below, forming a ligated probe which contains both oligonucleotide elements, and has the sequence-specific polymer chain and a reporter attached at opposite ends of the joined oligonucleotides. It will be recognized that the condition of abutting bases in the two probes can also be produced, after hybridization of the probes to a target region, by removing overlapping deoxyribonucleotides by exonuclease treatment.

Detailed Description Paragraph Right (34):
The second probe element is preferably labeled, for example, at its 3' end, with detectable reporter, such as reporter F indicated at 48 in FIG. 1C. Preferably the reporter is an optical reporter, such as a fluorescent molecule which can be readily detected by an optical detection system. A number of standard fluorescent labels, such as FAM, JOE, TAMRA, and ROX, which can be detected at different excitation wavelengths, and methods of reporter attachment to oligonucleotides, have been reported (Applied Biosystems, Connell).

Detailed Description Paragraph Right (35):
In one embodiment, each probe includes two second probe elements, one element having an end-subunit base sequence which can basepair with a wildtype base in the target sequence, and a second element having an end-subunit base sequence which can basepair with an expected mutation in the sequence. The two alternative elements are labeled with distinguishable reporters, allowing for positive identification of wildtype or mutation sequences in each target region, as will be described in Section III below. Alternatively, the two second probe elements (e.g., oligonucleotides) may have the same reporters, and the first probe elements have polymer chains which impart to the two probe elements, different ratios of charge/translational frictional drag, allowing the two target regions to be distinguished on the basis of electrophoretic mobility.

Detailed Description Paragraph Right (37):
Primer element 52, which is representative of the first primer elements in the probe composition, is composed of an oligonucleotide primer element 62 which preferably includes at least 7-15 bases, for requisite basepair specificity, and has a base sequence which is complementary to a 3'-end portion of region 56 in one of the two target strands, in this case, strand 58.

Detailed Description Paragraph Right (38):
The oligonucleotide primer is derivatized, at its 5' end, with a polymer chain 64 composed of N preferably repeating units 66, substantially as described with respect to chain 27 formed from units 28. As described with respect to probe 20, the polymer chain in the first probe element imparts a ratio of charge/translational frictional drag which is distinctive for each sequence-specific primer element in the composition.

Detailed Description Paragraph Right (39):
Second primer element 54, which is also representative of the second probe elements in the probe composition, is composed of an oligonucleotide primer element 68 which also preferably includes at least 7-15 bases, for requisite basepair specificity, and has a base sequence which is complementary to a 5' end portion of the opposite strand-in this case, strand 60, of the duplex DNA forming region 56. The second primer element may be labeled with a detectable reporter, as described above. Alternatively, labeling can occur after formation of amplified target sequences, as described below.

Detailed Description Paragraph Right (43):
FIG. 2 illustrates one method for preparing PEO chains having a selected number of HEO units. As shown in the figure, HEO is protected at one end with dimethoxytrityl (DMT), and activated at its other end with methane sulfonate. The activated HEO can then react with a second DMT-protected HEO group to form a DMT-protected HEO dimer. This unit-addition is carried out successively until a desired PEO chain length is achieved. Details of the method are given in Example 1.

Detailed Description Paragraph Right (44):
Example 2 describes the sequential coupling of HEO units through uncharged bisurethane tolyl groups. Briefly, with respect to FIG. 3, HEO is reacted with 2 units of tolylene-2,4-diisocyanate under mild conditions, and the activated HEO is then coupled at both ends with HEO to form a bisurethane tolyl-linked trimer of HEO.

Detailed Description Paragraph Right (45):
Coupling of the polymer chains to an oligonucleotide can be carried out by an extension of conventional phosphoramidite oligonucleotide synthesis methods, or by other standard coupling methods. FIG. 4A illustrates the coupling of a PEO polymer chain to the 5' end of an oligonucleotide formed on a solid support, via phosphoramidite coupling. FIG. 4B illustrates the coupling of the above bisurethane tolyl-linked polymer chain to an oligonucleotide on a solid support, also via phosphoramidite coupling. Details of the two coupling methods are given in Examples 3B and 3C, respectively.

Detailed Description Paragraph Right (52):
FIG. 6 shows an electropherogram of fluorescent-labeled 24-base oligonucleotide probes which are either underivatized (peak 1), or derivatized at their 5' ends with a 1, 2, or 4 phosphate-linked HEO subunits (peaks 2, 3, and 4, respectively). The probes were prepared as described in Example 4, and capillary electrophoresis was carried out in a buffer medium under the conditions detailed in Example 6.

Detailed Description Paragraph Right (58):
Following probe binding to the target polynucleotide, the probes are treated to selectively modify probes bound to the target sequences in a sequence-specific manner, to produce modified labeled probes, each having a distinctive charge/translational frictional drag ratio. This modifying step may involve joining probe elements by ligation, such as enzymatic ligation, across an expected mutation site, primer-initiated amplification of selected target sequences, probe extension in the presence of labeled nucleoside triphosphate molecules, enzymatic cleavage of a probe bound to a target region, or probe capture on an immobilized target, as detailed in Subsections A-E below.

Detailed Description Paragraph Right (66):
One of the probes in the composition, indicated at 80, includes a pair of probe elements 80a, 80b whose sequence are complementary to the corresponding subregions 74a, 74b, respectively in region 74 of the target polynucleotide i.e., the probe element sequences correspond to those of the "-" strand of the R.sub.2 region of the

target. In particular, the probe elements have end-subunits A and G bases which, when the elements are hybridized to complementary subregions of region 74, as shown, are effective to form Watson-Crick base pairing with adjacent bases T and C in the target region.

Detailed Description Paragraph Right (67):
Another of the probes in the composition, indicated at 82, includes a pair of probe elements 82a, 82b whose sequence are complementary to the corresponding subregions 76a, 76b, respectively in region 76 of the target polynucleotide. In this case, the probe elements have end-subunits A and C bases which, when the elements are hybridized to complementary subregions of region 76, as shown, are effective to form Watson-Crick base pairing with adjacent bases T and G bases in the wildtype target region. However, in the example shown, a T to G mutation prevents Watson-Crick base pairing of the A end-subunit to the associated target base.

Detailed Description Paragraph Right (68):
Following annealing of the probe elements to corresponding target sequences, the reaction mixture is treated with ligating reagent, preferably a ligase enzyme, to ligate pairs of probe elements whose confronting bases are base-paired with adjacent target bases. Typical ligation reaction conditions are given in Example 7A. The ligation reaction is selective for those probe elements whose end subunits are base-paired with the target bases. Thus, in the example illustrated, the probe elements 80a, 80b are ligated, but probe elements 82a, 82b are not.

Detailed Description Paragraph Right (69):
It can be appreciated that the ligation reaction joins an oligonucleotide carrying a sequence-specific polymer chain to an oligonucleotide carrying a detectable reporter, selectively forming modified, labeled probes, such as probe 84, composed of an oligonucleotide labeled at one end with a probe-specific polymer chain and at its other end with a detectable (fluorescent) reporter.

Detailed Description Paragraph Right (70):
Denaturing the target-probe complexes, as illustrated in FIG. 7D, releases a mixture of ligated, labeled probes, corresponding to wildtype target sequences, and non-ligated probe elements corresponding to point mutations at or near probe element end subunits. Each ligated, labeled probe has a polymer chain which imparts to that probe, a distinctive ratio of charge/translational frictional drag, as discussed above.

Detailed Description Paragraph Right (87):
FIGS. 12A and 12B illustrate the method. The figure shows the two separate strands 90, 92 of a normally double-stranded target polynucleotide 94 having at least one, and typically a plurality of regions, such as region 96, to be amplified. The target is reacted with a probe composition whose probes each consist of a pair of primer elements, such as primer elements 52, 54, in probe 50 described above with respect to FIG. 1C. FIG. 12A shows a probe 98 composed of primer elements 100, 102. Primer element 100 consists of an oligonucleotide primer 104 designed for hybridization to a 3' end of one strand of region 96, which carries at its 5'-end, a selected-length polymer chain 106, similar to above-describe primer element 52. Element 102 is an oligonucleotide primer designed for hybridization to a 5' end of the opposite strand region 96, which carries a fluorescent reporter at its 5'-end.

Detailed Description Paragraph Right (93):
In another application, it may be desired to assay which of a number of possible primer sequences, e.g., degenerate sequences, is complementary to a gene sequence of interest. In this application, the probe composition is used to amplify a particular sequence. Since each primer sequence will have a distinctive polymer chain, the primer sequence complementary to the sequence end regions can be determined from the migration characteristics of labeled probes. As with the other applications discussed above, the method may involve including in the fractionated probe mixture, a series of oligonucleotides derivatized with polymer chains of known sizes, and labeled different reporters groups that are carried on the test probes, to provide migration-rate standards for the electrophoretic separation.

Detailed Description Paragraph Right (96):

FIGS. 15A and 15B illustrate another method for modifying PCR-generated target fragments, such as double-stranded fragment 130, composed of strands 132, 136. In the embodiment illustrated, strand 132 has been fluorescent-labeled with a reporter 134 at one fragment end during amplification. The fragment strand can be reporter labeled by a variety of methods, such as by nick translation or homopolymer tailing in the presence of labeled dNTP's, or by PCR amplification using a reporter-labeled primer.

Detailed Description Paragraph Right (101):
A third general method for forming labeled probes, in accordance with the method of the invention, is illustrated in FIGS. 14A and 14B. In this method, a single-stranded target polynucleotide, such as shown at 120 in the figures, is reacted with a probe composition containing a plurality of probes, such as probe 122 which are designed for base specific binding to selected regions of the target. Probe 122, which is representative, is like probe 20 in FIG. 1A, and includes an oligonucleotide having a free 3'-end OH group and a selected-length polymer chain carried at its 5' end.

Detailed Description Paragraph Right (102):
After binding the probes to the target, the probes are treated with DNA polymerase I, in the presence of at least one reporter-labeled dNTP, as shown. Dye-labeled dNTPs can be synthesized from commercial starting materials. For example, amino 7-dUTP (Clontech, Palo Alto, Calif.) can be reacted with fluorescein NHS ester (Molecular Probes, Eugene, Oreg.) under standard coupling conditions to form a fluorescein-labeled dUTP. The polymerase is effective, in the presence of all four nucleoside triphosphates, to extend the 3' end of target-bound probes, incorporating one or more labeled nucleotides, as indicated at 128, to form the desired modified, labeled probes having distinctive polymer chains associated with each different-sequence probe, characteristic of each probe sequence. Alternatively, in the above example, fluorescein may be coupled to the modified nucleotide, e.g., amino-7-dU, after incorporation into the probe. Each of the different-sequence modified, labeled probes has a distinct ratio of charge/translational frictional drag by virtue of its distinctive polymer chain.

Detailed Description Paragraph Right (108):
In another embodiment using the cleavage mode of generating labeled probe, probe modification is accomplished during extension of a primer annealed to the target polynucleotide upstream from (beyond the 5' end of) the annealed probe. This extension is produced by a DNA polymerase also incorporating a 5' to 3' exonuclease activity (Holland). The method is illustrated in FIG. 19A which shows a target polynucleotide 222 with a sequence region 224 of interest. The probes in this method are exemplified by probe 226 which contains a binding polymer 228 having a subunit 229 adjacent the polymer's 5' end. Attached to this subunit are a polymer chain 230 and a labeled probe 232 (which may be derivatized to the free end of the polymer chain). Also shown in the figure is a primer 234 which is designed for sequence specific binding to the target, upstream of the region 224.

Detailed Description Paragraph Right (109):
In practicing the method, the sequence-specific probes and a set of primers, such as primer 234, are reacted with the target polynucleotide under hybridization conditions, to bind associated probes and upstream primers to different-sequence regions of the target. The target and attached probes are now treated with the above polymerase in the presence of all four nucleoside triphosphates, resulting in extension of the primer in a 5' to 3' direction, as indicated by x's in FIG. 19B. As the polymerase reaches the 5' end of the adjacent probe, it cleaves off the 5' end subunits from the probe. As shown in FIG. 19B, cleavage of the subunit 229 from the probe releases a labeled probe 236 composed of base 229, reporter 232, and polymer chain 230 which imparts to the labeled probe, a distinct ratio of charge/translational frictional drag.

Detailed Description Paragraph Right (110):
It will be recognized by one skilled in the art of molecular biology that many variants of the cleavage mode are practical; using exonuclease activities not linked to polymerase activities (e.g., the N-terminal selective cleavage fragment from E. coli polymerase I and the exonuclease of bacteriophage .lambda.), using the 3'.fwdarw.5' proofreading exonuclease activities of certain DNA polymerases (in which case the polymer chain 198 and the reporter F preferably are attached to the 3' end of

the probe, and this 3' end comprises one or more nucleotides mismatched to the template polynucleotide 188 of FIG. 17A), or using any of a wide range of sequence-specific endonucleases such as the restriction endonucleases. In all of these cases, the preferred embodiment locates the reporter and the polymer chain on the same side of the cleavage site(s), such that they remain covalently linked subsequent to cleavage. Additional polymer chains may or may not be added to the probe on the opposite side of the cleavage site(s) from the reporter in order to optimize the resolution of labeled probes from unlabeled probes.

Detailed Description Paragraph Right (155):
DMT-protected phosphoramidite HEO units from Example 1B were added to the 5' end of the oligo from Example 4A by standard phosphoramidite chemistry on solid support, yielding the composition 16 in FIG. 5. One to four units were added on in separate reactions. The resulting HEO modified oligos were cleaved from the solid support (Compound 17, FIG. 5) as above, and labeled with FAM and purified (Compound 18, FIG. 5), also as described above.

Detailed Description Paragraph Right (157):
A 25 base oligonucleotide having the sequence presented as SEQ ID NO:3 was made as described in Example 4A. DMT-protected phosphoramidite HEO units were added to the 5' end of this 25 mer and purified as described in Example 4B.

Detailed Description Paragraph Right (162):
A DNA mixture containing the four 26 mer oligonucleotides derivatized with 0, 1, 2, or 4 phosphate-linked HEO units, prepared as in Example 4, was diluted with 89 mM tris-borate buffer, pH 7.6, to a final DNA concentration of about 10.sup.-8 M. About 2 nanoliters of the DNA solution was drawn into the cathodic end of the tube by electrokinetic injection.

Detailed Description Paragraph Right (173):
(1) SEQ ID NO:3 derivatized at its 5' end with a either a 2 or 4 unit DEO (dodecyl ethylene oxide) polymer chains, according to synthetic methods described in Example 4, except in this case the units are 12 mers (2 or 4 12mers) of ethylene oxide;

Detailed Description Paragraph Right (176):
Probes 1 and 2 are designed to span a portion of one strand of the F508 region of the cystic fibrosis gene, as in Example 7. Probes 3 and 4 are designed to span the same portion of the F508 region of the opposite strand of the gene. Ligase chain reaction was performed according to published methods (Winn-Deen). Briefly, LCR assays were carried out in 20 mmol/L Tris.cndot.HCl buffer, pH 7.6, containing 100 mmol of K.sup.+, 10 mmol of Mg.sup.2+, 10 mmol of dithiothreitol, 1 mL of Triton X-100, and 1 mmol of NAD.sup.+ per liter. Each 100 .mu.L of reaction mixture contained 1 pmol of each of the four oligonucleotides and 15 U of thermal-stable ligase (Epicentre Technologies, Madison, Wis.). To mimic the complexity of the human genome, we added 4 .mu.g of herring sperm DNA to each reaction mixture. Reactions were carried out in 100-.mu.L aliquots overlayed with 100 .mu.L of mineral oil in Thin Walled Gene-Amp.RTM. (Perkin-Elmer Cetus, Norwalk, Conn.) reaction tubes. All LCR reactions were run in a Perkin-Elmer Cetus model 9600 thermal cycler for 30 cycles of 94.degree. C. (10S) and 60.degree. C. (2 min). At the end of the cycling protocol, the reactions were cooled to 4.degree. C.

CLAIMS:

3. The method of claim 1, wherein (i) each sequence-specific probe includes first and second probe elements having first and second oligonucleotides effective to bind to adjacent regions of a target sequence, where one of the oligonucleotides is derivatized with said polymer chain, (ii) said reacting is effective to bind both oligonucleotides to its specific target sequence, (iii) said treating includes ligating the oligonucleotides bound to the target polynucleotide under conditions which are effective to ligate the end subunits of target-bound oligonucleotides when their end subunits are base-paired with adjacent target bases, to form ligated probes, and releasing the ligated probe from the target polynucleotide, and (iv) the polymer chain attached to each different-sequence first oligonucleotide is effective to impart to the modified, labeled probe, a distinctive ratio of charge/translational frictional drag.

8. The method of claim 1, wherein (i) each sequence-specific probe includes first and second primer elements having first and second sequence-specific oligonucleotides effective to hybridize with opposite end regions of complementary strands of a target polynucleotide segment, respectively, where the oligonucleotide in the first primer element is derivatized with such probe-specific selected-polymer chain, (ii) said reacting is effective to bind both primer oligonucleotides to opposite end regions on complementary strands of the target polynucleotide, (iii) said treating is effective to amplify the target segment by primer-initiated polymerase chain reaction, and (iv) the polymer chain attached to each different-sequence first oligonucleotide is effective to impart to the amplified target sequences, a distinctive ratio of charge/translational frictional drag.

14. The method of claim 1, wherein the binding polymers are oligonucleotides, each sequence-specific probe includes (i) an oligonucleotide binding polymer having a 5' end, Where said polymer chain and a reporter label are attached to an oligonucleotide subunit adjacent said 5' end, and (ii) a primer designed for sequence-specific binding to the target upstream of said binding polymer, and said treating includes exposing immobilized probe to a DNA polymerase having a 5' to 3' exonuclease activity under conditions effective to extend said primer toward said binding polymer, said exposing being effective to enzymatically cleave said adjacent subunit from the binding polymer, forming a labeled probe whose polymer chain imparts to the probe, a distinctive charge/translational frictional drag.

End of Result Set

☐ Generate Collection

L9: Entry 3 of 3 File: USPT Apr 22, 1997

DOCUMENT-IDENTIFIER: US 5622824 A
TITLE: DNA sequencing by mass spectrometry via exonuclease degradation

Brief Summary Paragraph Right (4):
Apart from the time factor the biggest limitations of all methods involving PAGE as an integral part, however, is the generation of reliable sequence information, and the transformation of this information into a computer format to facilitate sophisticated analysis of the sequence data utilizing existing software and DNA sequence and protein data banks. With standard Sanger sequencing 200 to 500 bases of unconfirmed sequence information could be obtained in about 24 hours; with automatic DNA sequencers this number could be multiplied by approximately a factor of 10 to 20 due to processing several samples simultaneously. A further increase in throughput could be achieved by employing multiplex DNA sequencing [G. Church et al., Science, 240, 185-188 (1988); K oster et al., Nucleic Acids Res. Symposium Ser. No, 24, 318-21 (1991)] in which, by using a unique tag sequence, several sequencing ladders could be detected one after the other from the same PAGE after blotting, UV-crosslinking to a membrane, and hybridizations with specific complementary tag probes. However, this approach is still very laborious, often requires highly skilled personnel and can be hampered by the use of PAGE as a key element of the whole process.

Detailed Description Paragraph Right (10):
The solid supports can be of a variety of materials and shapes, such as beads of silica gel, controlled pore glass, cellulose, polyacrylamide, sepharose, sephadex, agarose, polystyrene and other polymers, or membranes of polyethylene, polyvinylidendifluoride (PVDF) and the like. The solid supports can also be capillaries, as well as frits from glass or polymers. Various 3' exonucleases can be used, such as phosphodiesterase from snake venom, Exonuclease VII from E. coli, Bal 31 exonuclease and the 3'-5' exonuclease activity of some DNA polymerases exerted in the absence of dNTPs, as for example T4 DNA polymerase.

Detailed Description Paragraph Right (13):
In another embodiment of the invention the unknown DNA sequence (target DNA) is inserted into a double-stranded cloning vector (FIG. 2) or obtained in double-stranded form, as for example by a PCR (polymerase chain reaction) process [PCR Technology, (1989) supra]. The DNA to be sequenced is inserted into a cloning vector, such as ligated into the Not I site as illustrated in FIG. 2. Adjacent to the A boundary there can be located another cutting restriction endonuclease site, such as an Asc I endonuclease cleavage site. The double-stranded circular molecule can be linearized by treatment with Asc I endonuclease and ligated to a solid support using a splint oligodeoxynucleotide (and ligase) as described above which restores the Asc I restriction site (Tr.sup.0 ds and Tr.sup.0' ds). The strand which is not immobilized can be removed by subjecting the double stranded DNA to standard denaturing conditions and washing, thereby generating single-stranded DNAs immobilized to the solid support (Tr.sup.0 ss and Tr.sup.0' ss). Since the unknown double-stranded DNA sequence can be ligated in either orientation to the support there can exist two non-identical 3' termini (+ and - strand) immobilized, which can result in ambiguous sequencing data. The immobilized fragment which carries the vector DNA sequence at the 3' end (Tr.sup.0' ss) can be protected from 3' exonuclease degradation during the sequencing process by, for example, annealing with an oligodeoxynucleotide complementary to the 3' end of the strand to be protected. As there can only be hybridization at one 3' terminus, i.e. to the wrong single-stranded DNA with (-) strand information (Tr.sup.0' ss), some alpha-thio dNTP's can be incorporated into the immobilized (-) strand via treatment with a DNA polymerase and completely protect that strand from exonucleolytic degradation [P. M. J. Burgers and F. Eckstein, Biochemistry, 18, 592 (1979); S.

Labeit, H. Lehrach and R. S. Goody, DNA, 5, 173 (1986); S. Labeit, H. Lehrach and R. S. Goody in Methods in Enzymology, Vol. 155, page 166 (1987), supra]. If desired, after incorporation of exonuclease resistant nucleotides, the oligonucleotide primer may be removed by a washing step under standard denaturing conditions. The immobilized single-stranded DNAs are transferred to the sequencing reactor (FIG. 9) and the sample with the unknown sequence at the 3' end is degraded by an exonuclease in a stepwise manner. The liberated nucleotides, or optionally modified nucleotides, are continuously fed into the mass spectrometer to elucidate the sequence.

Detailed Description Paragraph Right (18):
Mass-modified nucleotides can be incorporated by way of mass modified nucleoside triphosphates precursors using various methods. For example, one can begin with the insert of the target DNA sequence in a single-stranded cloning vector by having a "primer" and a "stopper" oligonucleotide bound to the complementary vector sequences located at the A and B boundary of the insert DNA respectively (FIG. 3) and a template directed DNA polymerase, preferentially one lacking the 3'-5' and 5'-3' exonuclease activity such as Sequenase, version 2.0 (US Biochemicals, derived from T7 DNA polymerase), Taq DNA polymerase or AMV reverse transcriptase. In the illustrative embodiment, the unknown DNA sequence has been inserted in a restriction endonuclease site such as Not I. Adjacent to the A boundary another restriction endonuclease site, such as the Asc I site, can be located within the primer binding site such that the partly double-stranded circular DNA can be cleaved at the unique Asc I site and the mass-modified (-) strand (t.sup.3 in FIG. 3) isolated by standard procedures (i.e. membrane filtration, molecular sieving, PAGE or agarose gel electrophoresis) and, if desired, coupled to a solid support via a splint oligonucleotide restoring the Asc I site in double-stranded form for ligation by T4 DNA ligase (FIG. 3). After having removed the splint oligonucleotide the immobilized single-stranded DNA fragment with its B' boundary at the 3' end (i.e. Tr.sup.3) is ready for exonuclease mediated mass spectrometric sequencing. In another illustrative embodiment, the same primer can be used even when the vector has no complementary Asc I site. Although the primer will not hybridize with its 5' terminal sequence to the vector as is shown in FIG. 3, it will nevertheless allow the covalent attachment of the single-stranded mass-modified DNA to the solid support using the same splint oligonucleotide as described above. In yet another embodiment the primer can carry a non-restriction site sequence information at its 5' end, which may or may not be complementary to the opposite vector sequence, but is complementary to a specific splint oligodeoxynucleotide which allows the covalent attachment to the solid support. The latter two procedures do not require cleavage with a restriction endonuclease and separation of the strands. The reaction mixture obtained after enzymatic synthesis of the mass-modified (-)strand can be directly joined to the solid support and the circular vector DNA and the stopper oligonucleotide can be removed under denaturing conditions. In yet another embodiment, the generation of a set of ordered deletions of the target DNA sequence information and the incorporation of mass modified nucleotides can be combined by terminating the DNA polymerase reaction at different time intervals (i.e. t.sup.0, t.sup.1, t.sup.2, t.sup.3, FIG. 3) to generate a ladder of mass-modified (-) strands. In case the 3' termini of each time point are too heterogeneous for mass spectrometric exonuclease sequencing a circularization and cloning step as described above can be included.

Detailed Description Paragraph Right (22):
The mass-modification of the immobilized strand starting with the unknown DNA insert in a double-stranded vector (FIG. 4A) can be introduced starting with a situation similar to Tr.sup.0 ds in FIG. 2. However, a 5' phosphorylated exonuclease III resistant splint oligonucleotide (i.e. 2',3' dideoxy) is ligated to the (-) strand allowing a unilateral digestion of the (+)strand with exonuclease III (FIG. 4A). The mass-modifications are then introduced by a filling-in reaction using template dependent DNA polymerases such as Sequenase, version 2.0 (US Biochemicals), Taq DNA polymerase or AMV reverse transcriptase and appropriate mass-modified dNTPs. In another embodiment one can start with a situation similar to Tr.sup.0 ss in FIG. 2 and by using a (-) primer designed to bind outside the A boundary at the 3' end of the (+)strand, synthesize a mass-modified (-) strand employing mass-modified dNTPs and a DNA dependent DNA polymerase as described above. In one embodiment, there can be a short stretch of sequence between the Not I and the Asc I site to allow this primer to hybridize effectively. This approach can also be carried out by generating a mass modified (+) strand starting with Tr.sup.0' ss (FIG. 4B). The newly synthesized (+) strand can be isolated from the (-) strand solid support, such as by denaturation, and

immobilized via the 5' sequence information of the primer and a splint oligonucleotide which is in part complementary to this and to an oligonucleotide sequence already attached to another solid support (FIG. 4B). After ligation (i.e. with T4 ligase) the splint oligonucleotide is removed and the immobilized mass-modified single-stranded (+) DNA is transferred to the sequencing reactor (FIG. 9) and contacted with an exonuclease, such as T4 DNA polymerase in solution, for mass spectrometric sequence determination via the released mass-modified nucleotides.

Detailed Description Paragraph Right (54):
The immobilized nucleic acids can be cleaved from the solid support by treatment with trypsin.

Other Reference Publication (8):
Beck et al. "Applications of Dioxetane Chemiluminescent Probes to Molecular Biology" Analytical Chemistry 1990, vol. 17, No. 21, pp. 2258-2270.

Other Reference Publication (11):
Brumbaugh et al. "Continous, on-line DNA sequencing using oligodeonucleotide primers with multiple fluorophores" PNAS 1988, vol. 85, pp. 5610-5614.

Other Reference Publication (42):
Prober et al. "A System for Rapid DNA Sequencing with Fluorescent Chain-Terminating Dideoxynucleotides" Science Research Articles 1987, vol. 238, pp. 336-341.